



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>7</sup> : <b>C12Q 1/68, G01N 33/50</b>		A1	(11) International Publication Number: <b>WO 00/37681</b> (43) International Publication Date: <b>29 June 2000 (29.06.00)</b>
(21) International Application Number: <b>PCT/US99/29856</b>		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: <b>17 December 1999 (17.12.99)</b>			
(30) Priority Data: <b>60/112,790 18 December 1998 (18.12.98) US</b>			
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(54) Title: BIOASSAY FOR IDENTIFYING ESTROGEN RECEPTOR- $\beta/\alpha$  SELECTIVE MODULATORS

## (57) Abstract

The present invention provides novel assay methods for identifying compounds that selectively activate estrogen receptors (ER) of the alpha or beta subtype. In particular, the results from two assays, one measuring ER- $\beta$  activity and the other measuring ER- $\alpha$  activity are interpreted. The assay measuring ER- $\beta$  activity uses cells comprising endogenous metallothionein-II as well as a DNA plasmid comprising a polynucleotide encoding human ER- $\beta$ . The assay monitors expression of metallothionein-II-mRNA in said cells, wherein the level of metallothionein-II expression is regulated when a potential ligand binds to ER- $\beta$ . The assay measuring ER- $\alpha$  activity uses cells comprising ER- $\alpha$  as well as DNA plasmid comprising a reporter gene linked to an estrogen response element. The assay monitors expression of the reporter gene, wherein the level of reporter gene is regulated when a potential ligand binds to ER- $\alpha$ . Compounds which modulate activity in one assay but have little or no activity in the other assay are defined as estrogen receptor subtype selective.

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**BIOASSAY FOR IDENTIFYING ESTROGEN RECEPTOR- $\beta/\alpha$  SELECTIVE MODULATORS**

**Technical Field**

5       The present invention relates to hormone receptors, and, more particularly to methods for identifying compounds that selectively activate estrogen receptors (ER) of the alpha or beta subtype, as well as a test kit for use in the methods.

10      **Background of the Invention**

Proteins which regulate gene expression are essential for cell function. A well-studied family of gene regulatory proteins is the steroid hormone receptor superfamily. These receptor proteins enable cells to respond to various hormones by activating or repressing specific genes.

15     One member of this family is the ER. Estrogens are classically known as important hormones in sexual development and reproductive function. It is well known that estrogens affect cell proliferation and differentiation in target tissues by binding to ERs in target cells. Estrogen replacement therapy is a well established treatment for prevention and/or amelioration  
20     of osteoporosis in postmenopausal women (Sagraves, 1995; Lobo, 1995) because these compounds have been demonstrated to prevent bone loss and fractures in women. Additionally, estrogen replacement therapy has been associated with a decreased mortality from cardiovascular disease. Finally, ongoing studies suggest that estrogens may provide

benefit to the central nervous system with regard to cognitive improvement and a decrease in Alzheimer's disease.

The classic ER, now designated ER- $\alpha$ , exhibits a modular structure with distinct domains involved in ligand binding, receptor dimerization,

5 DNA binding, and transcriptional activation (Green et al, 1986). The nucleotide sequence of the DNA binding domain is conserved among steroid hormone receptors. Kuiper et al (1996) exploited this similarity in an attempt to identify additional members of this family. The new member they discovered was recognized to be an ER because the amino acids in the DNA binding domain were almost identical to those of ER- $\alpha$ , , the *in vitro* translated protein specifically bound [ $^3$ H]-estradiol with nanomolar affinity and was able to regulate transcription from a simple estrogen response element. This protein has been designated ER- $\beta$  to distinguish it from the previously known form (now called ER- $\alpha$ ). Human

10 and mouse ER- $\beta$  have also been cloned Bhat et.al. 1998; Mosselman, 1996; Pettersson et al, 1997; Tremblay et al, 1997). Following ER- $\beta$ 's discovery, most work has focused on mapping the distribution of its mRNA in normal and neoplastic tissues, characterizing its binding affinity for a wide variety of ligands, and assessing its interaction with ER- $\alpha$ .

15 ER- $\beta$  mRNA is detectable by RT-PCR and *in situ* hybridization in a wide variety of tissues. While its distribution overlaps that of ER- $\alpha$ , some tissues express only one receptor type. For example both receptors are found in the uterus, ovary and pituitary, whereas ER- $\beta$  appears more

prominent in the prostate, lung and bladder (Kuiper et al, 1996). When examined by *in situ* hybridization, further distinctions between receptor distribution can be made. ER- $\beta$  mRNA is expressed in the epithelial cells of the rat prostate; ER- $\alpha$  is found in the stromal compartment (Kuiper et al, 1996). In the rat ovary, ER- $\beta$  appears in the granulosa cells; ER- $\alpha$  in the stroma (Shughrue et al, 1996; personal communication). In the rat hypothalamus, ER- $\beta$  but not ER- $\alpha$ , message is expressed in the paraventricular region, whereas both messages are seen in the preoptic area (Shughrue et al, 1996). Interestingly, significant species differences may exist in the relative levels of ER- $\beta$  and ER- $\alpha$  mRNA in certain organs. For example, ER- $\beta$  mRNA is highly expressed in the rat prostate, whereas more modest levels are detected in the human prostate. ER- $\beta$  predominates over ER- $\alpha$  in the rat prostate (Kuiper et al, 1996; Lau et al, 1998; Enmark et al, 1997), but the levels are more equal in the mouse prostate (Couse et al, 1997).

Several groups have characterized ER expression in tumor samples and cancer cell lines. Most work has been done in the breast where one study reports ER- $\beta$  mRNA using RT-PCR in 70% of forty breast biopsy samples (Dotzlaw et al, 1996). No correlation was noted between ER- $\alpha$  and ER- $\beta$  expression. Another study also reports heterogeneity of ER- $\alpha/\beta$  mRNA expression in breast tumor samples, with some tumors having only ER- $\alpha$  mRNA and others expressing mRNA for both receptor subtypes (Enmark et al, 1997). Among breast cancer cell lines, MDA MB 231 has

only ER- $\beta$  mRNA, and conflicting reports exist about the ER status of MCF-7 cells (Enmark, 1997; Kuiper et al, 1997; Vladusic et al, 1998; R. Henderson, unpublished observations).

Although the ligand binding domain of human ER- $\alpha$  and ER- $\beta$  are 5 59% identical at the amino acid level (Enmark et al, 1997), the binding affinity of 17 $\beta$ -estradiol is quite similar. Some compounds show marked selectivity for either ER- $\alpha$  or ER- $\beta$ . Genistein is a phytoestrogen which binds with approximately 10-25 fold higher affinity for ER- $\beta$  (Kuiper 1996, H. Harris unpublished observations). On the other hand raloxifene binds 10 about 20 fold better to ER- $\alpha$  than ER- $\beta$  (H. Harris, unpublished observations).

When ER- $\alpha$  and ER- $\beta$  are coexpressed , interaction can be measured by several methods including a mammalian two-hybrid system, glutathione S transferase pulldown and gel shift/supershift assays 15 (Cowley et al, 1997; Pettersson et al, 1997; Ogawa et al, 1998). Since these receptors can heterodimerize, estrogens' effect on tissues containing both receptors may be mediated by a complex interaction between ER- $\alpha$  and ER- $\beta$ .

One valuable tool in determining the function of ER- $\beta$  is the 20 estrogen receptor- $\alpha$  knockout (ERKO) mouse (Lubahn et al, 1993). Because these mice lack functional ER- $\alpha$ , they can help define the physiological roles of both ER- $\alpha$  and ER- $\beta$ . One study has compared the effectiveness of 17- $\beta$  estradiol treatment in ameliorating consequences of

artificially induced vascular injury to carotid arteries in wild type and ERKO mice (Iafrati et al, 1997). In both types of mice, pharmacological doses of 17- $\beta$  estradiol suppressed the increase in medial area and smooth muscle cell proliferation seen in the vehicle treated animals. It

5 is thought that these responses to endothelial denudation may narrow the lumen of the vessel, thus restricting blood flow. Because 17- $\beta$  estradiol was equally effective in ERKO as wild type mice, one interpretation is that ER- $\alpha$  is not necessary for this response. Because ER- $\beta$  mRNA is also expressed in these vessels, perhaps it mediates 17- $\beta$  estradiol's action.

10 However, direct evidence supporting this hypothesis is lacking. Another example of ERKO mice responding to 17- $\beta$  estradiol replacement was described in a recent poster and abstract (Pan et al, 1997, and personal communication). This study reports a loss of femoral bone mineral density and trabecular bone volume in ERKO mice after ovariectomy and

15 an increase in these parameters upon treatment with 17- $\beta$  estradiol (but not dihydrotestosterone). Again, based on indirect evidence, a suggestion is made that ER- $\beta$  has a physiological role.

Thus, while certain aspects of ER- $\beta$  have been characterized, the art fails to provide methods for identifying ligands which are functionally

20 selective for ER- $\alpha$  or ER- $\beta$ . Such an assay would be greatly advantageous to the pharmaceutical industry to uncover the possible therapeutic applications of the ER subtypes.

Summary of the Invention

The present invention provides method for screening a test compound that binds to an ER in a receptor binding assay, wherein said method detects ER- $\beta$  polypeptide-mediated transcription, said method comprising the steps of:

- (a) providing a cell which comprises at least one estrogen-induced DNA sequence encoding metallothionein (MT-II) and at least one DNA sequence encoding ER- $\beta$  polypeptide, wherein said receptor is transcriptionally active;
- 10 (b) contacting said cell with either said test compound which binds ER or a control; and
- (c) detecting the expression of said MT-II, wherein enhanced expression of said MT-II relative to a control indicates that said test compound has estrogen agonist activity.

15 This invention further provides a method for screening a test compound that binds to the ER in a receptor binding assay, wherein said method detects inhibition of ER- $\beta$  - polypeptide-mediated transcription, said method comprising the steps of:

- (a) providing a cell which comprises at least one estrogen-induced DNA sequence encoding metallothionein (MT-II) and at least one DNA sequence encoding ER- $\beta$  polypeptide, wherein said receptor is transcriptionally active;
- 20

(b) contacting said cell with one or more estrogen(s) in the presence of the test compound known to bind ER; and

(c) and detecting the expression of said MT-II, wherein decreased expression of said MT-II relative to the addition of one or more estrogen(s)

5 alone indicates that said test compound has estrogen antagonist activity

A method of screening test compounds to identify drug candidates which mimic estrogens' effect on ER- $\beta$ - or ER- $\alpha$ -mediated transcription is further provided wherein said method comprises the steps of:

(a) contacting said test compound with a plurality of:

10 (i) first cells comprising at least one DNA sequence encoding metallothionein (MT-II) and at least one DNA sequence encoding an ER- $\beta$  polypeptide, wherein said receptor is transcriptionally active and

(ii) second cells comprising an ERE reporter gene construct,

15 wherein said cells express ER- $\alpha$  polypeptide;

(b) identifying compounds which increase expression of MT-II in said first cells relative to control but have minimal effect on expression of said reporter gene in said second cells, wherein said compounds are considered ER- $\beta$  selective; or

20 (c) identifying compounds which increase expression of the reporter gene in said second cells relative to control but have minimal effect on expression of MT-II in said first cells, wherein said compounds are considered ER- $\alpha$  selective.

Lastly, the present invention provides a method of screening test compounds to identify drug candidates which inhibit estrogen's effect on ER- $\beta$ - or ER- $\alpha$ -mediated transcription, said method comprising the steps of:

- 5       (a) contacting said test compound in the presence and absence of one or more estrogen(s) with a plurality of:
  - (i) first cells comprising at least one endogenous DNA encoding a metallothionein (MT-II) gene and DNA encoding a ER- $\beta$  polypeptide, wherein said receptor is transcriptionally active and
  - (ii) second cells comprising an ERE reporter gene construct, wherein said cells express ER- $\alpha$  polypeptide; and
- 10      (b) identifying compounds which decrease expression of MT-II in said first cells relative to treatment with one or more estrogen(s) alone but have minimal effect on expression of said reporter gene in said second cells, wherein said compounds are considered ER- $\beta$  selective; or
- 15      (c) identifying compounds which decrease expression of the reporter gene in said second cells relative to treatment with one or more estrogen(s) alone but have minimal effect on expression of MT-II in said first cells, wherein said compounds are considered ER- $\alpha$  selective.
- 20

**Brief Description of the Figures**

Figure 1: RT-PCR amplification of ER- $\beta$  and ER- $\alpha$  from Saos -2 and LNCaPLN3 cells.

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Figure 2: Sequencing gel separating amplified cDNAs and 17- $\beta$  estradiol upregulation of fragment 6a.

10 Figure 3: (A) Nucleotide sequence of the regulated fragment and its alignment with human MT-II . (B) Translation of fragment sequence from first methionine to stop codon (\*)

15 Figure 4: Regulation of MT-II in two cell lines. To determine fold change in mRNA, MT-II signal was normalized to that of GAPDH, and compared to the control cells.

20 Figure 5: Dose response of 17- $\beta$  estradiol regulation of MT-II in Saos-2 cells. Result are shown for four different experiments and the EC<sub>50</sub> shown for each.

Figure 6: Time course of metallothionein-II regulation in Saos-2 cells. Data is normalized with GAPDH and fold change calculated from the vehicle control at time = 0hr.

10

Figure 7: Receptor specificity of MT-II regulation in Saos-2 cells.

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Figure 8: Regulation of MT-II in Saos-2 cells by various compounds.

Figure 9: Metallothionein-II regulation in cycloheximide-treated Saos-2 cells. (A) Measurement of protein synthesis during treatment with cycloheximide. (B) Whole cell ER binding assay after 8 hours of cycloheximide treatment. (C,D) Metallothionein-II regulation after 8 and 24 hours of treatment respectively.

10

Figure 10: MT is regulated by estrogens in the rat prostate.

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Figure 11: Induction of MT-II in the rat prostate requires at least two days of dosing.

20

Figure 12: Screening strategy for ligands which selectively activate ER- $\beta$

and/or ER- $\alpha$ .

Detailed Description of the Invention

The present invention provides an efficient way to screen large numbers of test compounds which selectively activate ERs of the  $\alpha$  or  $\beta$  subtype. These compounds may have desirable properties for either the treatment or the prevention of various diseases mediated by estrogens, 5 including but not limited to cancers (e.g. breast, ovarian, endometrial, prostate), endometriosis, osteoporosis and cardiovascular and central nervous system diseases.

#### Definitions

10 In the present description and claims, the following terms shall be defined as indicated below.

“hER $\beta$ L” is defined as the human form of ER- $\beta$  described in Example 1 (the cDNA or its translated protein product).

15 “Estrogen” is defined as any ligand that can function as an estrogen agonist.

“Estrogen agonist” is defined as a compound that substantially mimics 17- $\beta$  estradiol as measured in a standard assay for estrogenic activity, for example, cellular assays as described in Webb et al. (1992).

20 “Estrogen antagonist” is defined as a compound that substantially inhibits the effect of estrogen agonists as measured in a standard assay for estrogenic activity, for example, cellular as described in Webb et al. (1992)

"A functional ER" is defined as a receptor capable of transcriptional activation of endogenous or transfected genes as measured by changes in RNA, protein and/or downstream biological events.

"A nonfunctional ER" is defined as a receptor incapable of

5 transcriptional activation of endogenous or transfected genes as measured by changes in RNA, protein and/or downstream biological events. A "test compound" includes but is not limited to any small molecule compound, peptide, polypeptide, natural product, toxin with potential biological activity.

10 A "ligand" is intended to include any substance that interacts with a receptor.

"Transfection" is defined as any method by which a foreign gene is inserted into a cultured cell.

A "reporter" is defined as any substance that can be readily

15 measured and distinguished from other cellular components. The reporter may be the transfected receptor DNA, the transcribed receptor mRNA, an enzyme, a binding protein or an antigen.

A "cell" useful for the present purpose is one which has the ability to respond to signal transduction through a given receptor.

20 "A "receptor binding assay" is an assay measuring the amount of ligand specifically interacting with a receptor. The ligand can be a radioligand (e.g. conjugated to  $^3\text{H}$  or  $^{125}\text{I}$ ), a fluorescinated ligand (either

conjugated to a fluorochrome or possessing inherent fluorescence) or otherwise labeled so as to be detectable.

#### Description of the Assay

The present invention relies on the discovery that the expression of  
5 MT-II is selectively regulated by the interaction of a ligand with ER-β. .

In the methods of the invention, the regulation of MT-II activity may be used to provide a screening system that selectively detects both estrogen agonist or antagonist functional activity of a ligand following its interaction with ER-β.

10 The methods typically comprise cultured cells that express functional human ER-β and no, or a diminished amount of ER-α. Such cells include but are not limited to Saos-2 (ATCC HTB-85) and LNCaPLN3. Preferred cells for this purpose include cells which over-express ER-β, such as the cells described below which are recombinantly manipulated to over-express ER-β. The ER-β receptor may be modified in any way, such as in length; these modifications may result in increased ER-β selectivity and increased sensitivity of the assay.  
15

One of skill will recognize that various recombinant constructs comprising ER-β can be used in combination with any cell or line which  
20 lacks functional ER-α.

To screen a number of compounds for estrogen agonist activity, cells expressing ER-β are exposed to a test compound or a control solution (which is used to dissolve the test compound). The cells can be

exposed while either growing in separate wells of a multi-well culture dish or in a semi-solid nutrient matrix. After treatment for a suitable period of time, MT-II mRNA is measured. An estrogen agonist will increase MT-II mRNA when compared to treatment with the control solution alone.

5        To screen a number of compounds for estrogen antagonist activity, cells expressing ER- $\beta$  are exposed to one or more estrogens in the presence or absence of a test compound. The cells can be exposed while either growing in separate wells of a multi-well culture dish or in a semi-solid nutrient matrix. After treatment for a suitable period of time, MT-II

10      mRNA is measured. An estrogen antagonist will decrease MT-II mRNA when compared to treatment with the estrogen solution alone.

Estrogenic or antiestrogenic compounds identified in the assays of the invention can be used in standard pharmaceutical compositions for the treatment of cancer, as components of oral contraceptives, or any other application in which the modulation of estrogen activity is desired. The pharmaceutical compositions can be prepared and administered using methods well known in the art. The pharmaceutical compositions are generally intended for parenteral, topical, oral or local administration for prophylactic and/or therapeutic treatment. The pharmaceutical compositions can be administered in a variety of unit dosage forms depending upon the method of administration. For example, unit dosage forms suitable for oral administration include powder, tablets, pills, and capsules. Suitable pharmaceutical formulations for use in the present

invention are found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Philadelphia, Pa., 17th ed. (1985). A variety of pharmaceutical compositions comprising compounds of the present invention and pharmaceutically effective carriers can be prepared.

5

### Applications

The methods and compositions of the present invention can be used to identify compounds that interact with ER- $\beta$ , either to stimulate or to inhibit transcriptional activity. Such compounds include, without limitation, co-activator proteins, as well as estrogens and other steroids, steroid-like molecules, or non-steroid-like molecules that act as agonists or antagonists. 10 Screening methods can also be used to identify tissue-specific estrogens.

Identification of ER- $\beta$ -interactive compounds can be achieved by cell-free or cell-based assays. In one set of embodiments, purified ER- $\beta$  is contacted with a labeled ligand, such as, e.g., 17- $\beta$  estradiol, in the presence of test compounds to form test reactions, and in the absence of test compounds to form control reactions. The labeled moiety may comprise a radiolabel (such as, e.g.,  $^3\text{H}$  or  $^{125}\text{I}$ ) or a fluorescent molecule. Incubation is allowed to proceed for a sufficient time and under appropriate conditions to achieve specific binding, after which binding of labeled ligand 15 to ER- $\beta$  is measured (by monitoring, e.g., radioactivity, fluorescence, or fluorescence polarization). In one embodiment, the ligand binding domain of ER- $\beta$  produced in *E. coli* is adsorbed to the wells of a microtiter dish and incubated with [ $^3\text{H}$ ]-17 $\beta$  estradiol in the absence or presence of test 20

compounds. Alternatively, soluble receptor is incubated with the labeled ligand in the absence or presence of test compounds, and bound ligand is separated from free ligand, either by filtration on glass fiber filters or by using dextran-coated charcoal

5        Whole cell binding assays may also be used in which bound ligand is separated from free ligand by rinsing. Cells used in these assays may either contain endogenous receptor, or may overexpress the receptor subsequent to stable or transient transfection or infection of an ER- $\beta$  cDNA. Non-limiting examples of suitable cells include COS cells, HeLa cells, CHO cells,

10      human umbilical vein endothelial cells and yeast. Once a compound has been identified as an ER- $\beta$ -interactive compound by its binding activity, further in vivo and in vitro tests may be performed to determine the nature and extent of activity, i.e., as an agonist or antagonist (see below).

ER- $\beta$ -interactive compounds may also be identified using cell-based assays that measure transcriptional activation or suppression of endogenous or transfected estrogen-responsive genes. For example, agonists (such as, e.g., 17 $\beta$ -estradiol) block interleukin-1 $\beta$  induction of endogenous E-selectin in primary human umbilical vein endothelial cells that express ER- $\beta$ . Antagonists (such as, e.g., ICI-182780) block the agonist activity of 17 $\beta$ -estradiol. Non-limiting examples of other suitable endogenous estrogen-responsive promoter elements include those that regulate endothelin-1 (ET-1); HDL receptor (scavenger receptor type II); and enzymes involved in coagulation and fibrinolysis (such as, e.g., plasminogen

activator inhibitor-1 and complement C3). Any promoter element that responds to estrogen may be used as an appropriate target, including, e.g., the NF<sub>k</sub>B binding site or the apolipoprotein A1 gene enhancer sequence.

In one set of embodiments, appropriate host cells are transfected

- 5 with an expression vector encoding ER-β and the transfectants are incubated with or without one or more estrogens I in the presence or absence of test compounds. ER-β activity is assessed by measuring transcriptional activation. This may be achieved by detection of mRNA (using, e.g., Northern blot analysis, reverse transcriptase polymerase chain reaction, RNase protection assays) and/or by detection of the protein (using, e.g., immunoassays or functional assays). If activation of the target sequence initiates a biochemical cascade, downstream biological events may also be measured to quantify ER-β activity. ER-β -interactive compounds are identified as those that positively or negatively influence
- 10 target sequence activation.
- 15

- 20 In another set of embodiments, appropriate host cells (preferably, mammalian ) are co-transfected with an expression vector encoding ER-β and a reporter plasmid containing a reporter gene downstream of one or more ERE s. Transfected cells are incubated with or without an estrogen in the presence or absence of test compounds, after which ER-β activity is determined by measuring expression of the reporter gene. In a preferred embodiment, ER-β activity is monitored visually. Non-limiting examples of

suitable reporter genes include luciferase, chloramphenicol acetyl transferase (CAT), and green fluorescence protein.

Preferably, the methods of the present invention are adapted to a high-throughput screen, allowing a multiplicity of compounds to be tested in

5     a single assay. Candidate estrogens and estrogen-like compounds include without limitation diethylstilbestrol, genistein, and estrone. Other ER- $\beta$  - interactive compounds may be found in, for example, natural product libraries, fermentation libraries (encompassing plants and microorganisms), combinatorial libraries, compound files, and synthetic compound libraries.

10    For example, synthetic compound libraries are commercially available from Maybridge Chemical Co. (Trevillet, Cornwall, UK), Comgenex (Princeton, NJ), Brandon Associates (Merrimack, NH), and Microsource (New Milford, CT). A rare chemical library is available from Aldrich Chemical Company, Inc. (Milwaukee, WI). Alternatively, libraries of natural compounds in the

15    form of bacterial, fungal, plant and animal extracts are available from, for example, Pan Laboratories (Bothell, WA) or MycoSearch (NC), or are readily produced . Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means (Blondelle et al., 1996). ER- $\beta$  binding assays

20    according to the present invention are advantageous in accommodating many different types of solvents and thus allowing the testing of compounds from many sources.

Compounds identified as ER- $\beta$  agonists or antagonists using the methods of the present invention may be modified to enhance potency, efficacy, uptake, stability, and suitability for use in therapeutic applications, etc. These modifications are achieved and tested using methods well-known in the art.

### Examples

The present invention is further described by the following examples. The examples are provided solely to illustrate the invention by reference to specific embodiments. These exemplifications, while illustrating certain specific aspects of the invention do not portray the limitations or circumscribe the scope of the invention.

#### EXAMPLE 1: CONSTRUCTION OF ER- $\beta$ RECOMBINANT ADENOVIRUS

15       The coding sequence of human ER- $\beta$  was obtained as described in co-owned and co-assigned pending US Serial 08/906,365 entitled "Novel Human Estrogen Receptor  $\beta$ " filed August 5, 1997, and in Bhat et. al., (1998). Essentially, human testis Poly A + RNA (1  $\mu$ g, Clontech, Palo Alto CA) was mixed with 0.5  $\mu$ g oligo dT primer (GIBCO-BRL, Gaithersburg MD)

20       in a total volume of 10  $\mu$ l. The mixture was heated at 70°C for 10 minutes, and, after cooling on ice, was supplemented with 500  $\mu$ M of each deoxynucleoside triphosphate, 1X cDNA synthesis buffer, and 10 mM DTT to a final reaction volume of 20  $\mu$ l. The mixture was incubated at 42°C for 2.5 minutes and then supplemented with 1-2 units reverse transcriptase

(GIBCO-BRL, Gaithersburg MD), after which it was incubated at 45°C for 30 minutes and 50°C for 5 minutes. One-tenth of this mixture (approximately 2 µl) containing the cDNA template was then used in PCR amplification of ER-β using forward and reverse primers as described below.

5       The PCR primers designated in Serial No. 08/906,365 (supra) were used to amplify ER-β sequences in a reaction containing the following components: 2 µl of the cDNA template described above; 1X PCR buffer; 200 µM of each deoxynucleoside triphosphate, 2 units of Hot Tub DNA polymerase (Amersham, Arlington Heights IL), and 1µg of each of the  
10 forward and reverse primers. The reaction mixture was heated to 95°C for 2 minutes, annealed at 52°C for 1 minute, and amplified using 36 cycles, followed by an incubation at 72°C for 1.5 minutes.

A fragment of approximately 1500 bp in length was produced. The fragment digested with HindIII and XbaI (which cleave at sites present in  
15 the forward and reverse primer sequences, respectively, but not in the main body of the amplified cDNA sequence) and cloned into the corresponding sites of the pcDNA3 expression vector (Invitrogen, Carlsbad CA). This asymmetric cloning strategy places the 5' end of ER-β cDNA under the control of the viral CMV promoter in pcDNA3. This clone was designated  
20 "truncated hERβ" or hERβT.

To verify the amino terminal and upstream sequence of human ERβ, two independent approaches were taken, as described below.

(1) 10 µl of a human ovary 5'-Stretch cDNA library (Clontech, Palo Alto CA) was mixed with 50 µl of 1X K solution (1X PCR Buffer (GIBCO-BRL, Gaithersburg MD), 2.5 mM MgCl<sub>2</sub>, 0.5% Tween-20, 100 µg/ml Proteinase K), and the reaction mixture was incubated at 56°C 5 for 2 hours, then at 99°C for 10 minutes. Five µL of this reaction mixture were then used as template in a nested PCR reaction using the PCR primers designated in Serial No. 08/906,365 (supra). The reaction contained 1X KlenTaq PCR reaction buffer (40 mM Tricine-KOH, 15 mM KOAc, 3.5 mM Mg(OAc)<sub>2</sub>, 75 µg/ml bovine serum albumin); 0.2 µM of each dNTP; 0.2 µM 10 of each of the above primers, and 1 unit of KlenTaq Polymerase Mix (Clontech, Palo Alto CA). Touchdown PCR conditions were as follows: 5 cycles of 94°C for 2 seconds and 72°C for 4 minutes, followed by 30 cycles of 94°C for 2 seconds and 67°C for 3 minutes.

Excess nucleotides and primers were removed from first round PCR 15 reactions by purification using Wizard PCR columns (Promega, Madison WI). A second-round PCR reaction was then performed using 2 µl of the purified first-round reaction mixture using the PCR primers designated in Serial No. 08/906,365 (supra). The PCR reaction and cycling conditions were identical to those employed in the first round. The products were 20 cloned into pCR2.1 (Invitrogen, Carlsbad CA) and three resulting clones were sequenced. All three clones (designated L1, L2, and L3) contained ERβ inserts of different lengths, all of which were homologous to ERβ and to each other.

(2) A Marathon Ready thymus cDNA kit (Clontech, Palo Alto CA) for 5' rapid amplification of cDNA ends (RACE) was also used to isolated ER $\beta$  5' clones. In the first round of a nested PCR reaction, 5  $\mu$ l of human thymus Marathon-ready cDNA (Clontech, Palo Alto CA) was used as template and using the primers designated in Serial No. 08/906,365 (supra). The PCR reaction and cycling conditions were identical to those described in (1) above.

Excess nucleotides and primers were removed from the first round PCR reactions by purification over Wizard PCR columns (Promega, Palo Alto CA). A second round PCR reaction was performed using 2  $\mu$ l of the purified first round reaction using primers designated in Serial No. 08/906,365 (supra). The second round PCR reaction and cycling conditions were identical to those employed in the first round. The products were cloned into the pCR2.1 vector (Invitrogen, Carlsbad CA) and two clones were sequenced. The two clones contain insert sequences of different lengths that are homologous to ER $\beta$ , to each other, and to the sequences isolated from a human ovary cDNA library as described above.

All of the ER $\beta$  sequences isolated by methods (1) and (2) above contained 110 nucleotides corresponding to hER $\beta$ T sequences, as well as 228 additional nucleotides at the 5' end.

The hER $\beta$ T and 5' end sequences were joined and the resulting cDNA was cloned into pCDNA3 (Invitrogen, Carlsbad CA ) under the control of the cytomegalovirus IE promoter; this expression vector was designated "long

hER- $\beta$ " or hER $\beta$ L . Full length hER $\beta$ L cDNA sequence encodes a polypeptide having 530 amino acid residues as seen in SEQ ID No.1.

The hER $\beta$ L sequence contained an optimal translation initiation sequence CCACC immediately upstream to the initiation codon and the sequence

5 was under the control of the cytomegalovirus IE promoter. The coding sequence of hER $\beta$ L was then transferred into an Ad5 $\Delta$ E1a vector plasmid containing adenovirus sequences from map unit 0-17 with an deletion of E1a region between map units 1.4 -9.1 (Davis AR et. al., 1985, Gluzman,Y. et. al.,1982). The hER $\beta$ L transcription unit in Ad5 $\Delta$ E1a

10 plasmid contained cytomegalovirus 1E promoter, Ad5 tripartite leader, coding sequence of hER $\beta$ L and SV40 late polyadenylation signal sequences. hER $\beta$ L in Ad5  $\Delta$ E1a plasmid was then linearized with BstEII enzyme and transfected along with Clal A fragment of Ad5 virus with E3 region deletion (80-88 map units) into 293 cells (transformed primary

15 human embryonic kidney, ATCC CRL 1573). Viral plaques generated by homologous recombination were isolated, amplified and characterized by restriction DNA analysis and cell lysis assay in A549 cells (human lung carcinoma, ATCC CCL 185). Confirmatory tests indicated that the recombinant Ad5 hER $\beta$ L virus contained the expected DNA fragments and

20 was replication defective. The virus was further purified by re-plaquing. The isolated plaques were amplified, tested and used as a seed stock to generate large amounts of the virus in 293 cells. The virus was titered in 293 cells by plaque assay and the stock contained 1.28 X10<sup>9</sup> PFU/ml.

**EXAMPLE 2: ASSESSMENT OF ENDOGENOUS LEVELS OF ER mRNA IN SAOS-2 AND LNCAPLN3 CELLS**

Unless otherwise noted, cell culture reagents were obtained from

5    Gibco BRL (Gaithersburg MD). LNCaPLN3 cells were grown in RPMI 1640 medium supplemented with 10% FBS, 2 mM GlutaMAX-1, 100 U/ml penicillin g, and 100 µg/ml streptomycin sulfate. Saos-2 cells (ATCC, Manassas VA) were maintained in monolayer culture using McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), 2 mM

10    GlutaMAX-1, 100 U/ml penicillin g, 100 µg/ml streptomycin sulfate.

**RT-PCR**

Total RNA was isolated from LNCaPLN3 and Saos-2 cells using TRizol (Gibco BRL, Gaithersburg MD) according to the manufacturer's directions. The samples were then treated with RNase-free DNase I

15    (Gibco BRL, Gaithersburg MD) at 1 unit/µg for 30 minutes at 37°C. RNA was purified from the reaction using RNeasy columns (Qiagen, Hilden Germany) and amount recovered estimated by UV spectrophotometry.

Reverse transcription reactions were performed on 0.5 µg of RNA in a 20 µl reaction. For ER-α the reaction contained 1x PCR Buffer (Gibco

20    BRL, Gaithersburg MD), 5 mM MgCl<sub>2</sub>, 1.25 µM ERα-specific reverse primer (5'-CCAGCAGCATGTCGAAGATC-3'), 0.5 µM GAPDH-specific reverse primer (5'-CACCTGTTGCTGTAGCCAAATTC-3'), 0.5 mM dNTPs, 20 units of RNasin (Promega, Madison WI) and 200 units of Superscript II Reverse Transcriptase (Gibco BRL, Gaithersburg MD). The

ER- $\beta$  reaction contained the same components as ER- $\alpha$  with the following exceptions: 2.5 mM MgCl<sub>2</sub> and 1.25  $\mu$ M ER $\beta$ -specific reverse primer (5'-GCAGAAGTGAGCATCCCTTTG-3'). A duplicate reaction which was identical in all reagents except that it did not contain Superscript II

- 5 Reverse Transcriptase was performed for each sample as a negative control to ensure that the RNA samples were not contaminated by DNA. Reactions were incubated at 42°C for 15 minutes followed by 5 minutes at 99°C and 5 minutes on ice prior to amplification.

PCR was initiated by adding 80 $\mu$ l of master mix containing ER- $\alpha$ -specific forward primer (5'-GGAGACATGAGAGCTGCCAAC-3') or ER- $\beta$ -specific forward primer (5'-CAGCATTCCCAGCAATGTCAC-3') and GAPDH-specific forward primer (5'-GACATCAAGAAGGTGGTGAAGCAG-3') directly to the 20 $\mu$ l reverse transcriptase reaction. Final concentration of reagents in the ER- $\alpha$  100 $\mu$ l PCR reaction was as follows:

- 15 0.25  $\mu$ M each ER-specific primer, 0.1 $\mu$ M each GAPDH primer, 1x PCR Buffer (Gibco BRL, Gaithersburg MD), 0.2 mM dNTPs, 2 mM MgCl<sub>2</sub> and 0.5 units of Taq DNA Polymerase (Gibco BRL, Gaithersburg MD). The ER- $\beta$  reaction contained the same amount of reagents except for 1 mM MgCl<sub>2</sub>. Two step PCR was carried out in a PE 9600 for 25 cycles as follows: 95°C for 30 sec, 64°C for 1.5 min. Samples were incubated at 64°C for 10 min after amplification.
- 20

Twenty microliters of each sample were separated using a 1.5% agarose gel and transferred to Hybond-N + (Amersham Pharmacia,

Piscataway NJ) by capillary alkali Southern Blotting in 0.4 N NaOH, 0.6M NaCl. Blots were pre-hybridized at 42°C for 30 min in Rapid-Hyb buffer (Amersham Pharmacia, Piscataway NJ). Oligonucleotide probes specific for ER- $\alpha$  (5'-TGAACCAGCTCCCTGTCTGCCAGGTTGGT-3'), ER- $\beta$  (5'-CCGCATACAGATGTGATAACTGGCGATGGA-3') and GAPDH (5'-GCTGTTGAAGTCACAGGAGACAACCTGGT-3') fragments were end-labeled with  $^{32}$ P- $\gamma$  ATP using Polynucleotide Kinase (Gibco BRL, Gaithersburg MD). Probes were added to the blot at  $3.0 \times 10^6$  CPM/ml and incubated at 42°C for 1 hour. ER and GAPDH hybridizations were done independently. Blots were washed once in 2x SSC, 0.1% SDS at room temperature for 15 min then twice in 0.2x SSC, 0.1% SDS at 42°C for 15 min. Blots were then exposed to film.

Saos-2 cells expressed endogenous ER- $\beta$  but not ER- $\alpha$  mRNA when assessed by PCR (Figure 1). As a positive control, GAPDH mRNA was coamplified in these reactions. Whereas both cell lines contained GAPDH mRNA, only the Saos-2 cells contained ER- $\beta$ . Similar results were obtained for the LNCaPLN3 cells (data not shown).

**EXAMPLE 3: DISCOVERY THAT ER- $\beta$  UPREGULATES MT-II- IN SAOS-2 AND LNCAPLN3 CELLS**

Cell culture and infection

Because message levels for ER- $\beta$  were low, Saos-2 and LNCaPLN3

5    cells were engineered to overexpress hER $\beta$ L by transient infection with a recombinant adenovirus (see Example 1) prior to differential display.

LNCaPLN3 and Saos-2 cells were cultured as described above.

Sixteen hours prior to infection, the cells were plated in phenol red-free RPMI 1640 medium supplemented with 10% charcoal/dextran-treated  
10    ("stripped") FBS (HyClone, Logan UT), 2 mM GlutaMAX-1, 100 U/ml penicillin g, 100  $\mu$ g/ml streptomycin sulfate. This medium was used for the remainder of the experiment.

Cells were infected with a 1/20 dilution of an Ad5 hER $\beta$ L virus (see Example 1) using 2% stripped FBS phenol red-free medium with  
15    antibiotics and GlutaMAX-1 for 2 hours at 37° C. Medium containing virus was aspirated and the cells were washed with medium. Fresh medium was added and the cells allowed to recover overnight at 37°C.

The following day, cells were treated with 10 nM 17 $\beta$ -estradiol or vehicle for 24 hours and total RNA prepared for differential display using  
20    the TRIzol reagent (Gibco BRL, Gaithersburg MD). To remove residual DNA, samples were treated with RNase-free DNase I (Gibco BRL, Gaithersburg MD) at 1 unit/ $\mu$ g for 30 minutes at 37°C. RNA was purified from the reaction using RNeasy columns (Qiagen, Hilden Germany) and amount recovered estimated by UV spectrophotometry.

Rapid Analysis of Differential Expression (RADE)*Reverse transcription (RT)*

Following DNase I treatment, six micrograms total RNA was incubated with 1x RT buffer (25 mM Tris-Cl, pH 8.3, 37.6 mM KCl, 3 mM MgCl<sub>2</sub> and 5 mM DTT, from Genhunter, Nashville TN), 20 µM dNTP's (dA, C, G and TTP 2'- deoxynucleoside 5' triphosphates, From Gibco BRL (Gaithersburg MD), 0.2 µM HT<sub>11</sub>C (oligonucleotide AAGCTTTTTTTTC) in a final volume of 600 µL. This reaction mixture was incubated at 65°C for five minutes to denature secondary structures, followed by a ten minute incubation at 37°C. At this time 30 µl Superscript II reverse transcriptase (200U/µl, Gibco BRL, Gaithersburg MD) was added to the reaction and incubation proceeded for 1 hr at 37°C. The enzyme was inactivated by heating at 75°C for five minutes. An aliquot of this reaction was then used for the second strand synthesis by PCR.

**15 Polymerase chain reaction (PCR)**

To 2µl of the RT reaction was added, 1x PCR buffer (10 mM Tris-Cl, pH 8.4, 100 mM KCl, 1.5 mM MgCl<sub>2</sub> and 0.001% gelatin), 2 µM dNTP's, 15 nM <sup>33</sup>P dATP (NEN, Boston MA), 1 unit AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk CT) and 1µM arbitrary primer 5'AAGCTTGCCATGG-3' for a total reaction volume of 20µl. This reaction mixture was then thermocycled using the following parameters:  
92°C for 2 min, 1 cycle

92°C for 15 sec, 40°C for 2 min, 72°C for 30 sec, 40

cycles

72°C for 5 min

*Gel electrophoresis*

5        Duplicate samples of PCR products were separated by gel electrophoresis on a 6% denaturing polyacrylamide gel (5.7% acrylamide, 0.3% bisacrylamide, 42% urea and 51% H<sub>2</sub>O) in 1x TBE buffer (0.1 M Tris, 0.09 M Boric Acid, 1 mM EDTA) for three hours at 2000 volts. The gel was then transferred to filter paper (Schleicher & Schuell, Keene NH),  
10      dried under vacuum at 80°C for one hour and exposed to X-ray film for twenty four hours.

One band, designated 6a, was apparently upregulated in both the Saos-2 and LNCaPLN3 cells by 17-β estradiol (Figure 2). The developed film was then superimposed over the dried gel and the band's corners  
15      were marked using a 22 gauge syringe needle. The gel slice within these boundaries excised with a razor blade and immersed in 100 µl H<sub>2</sub>O. This sample was boiled in a water bath for fifteen minutes, centrifuged for two minutes and the supernatant solution transferred to a new tube. Added to this sample was 5 µl of 10 mg/ml glycogen, 10µl of 3 M sodium acetate  
20      and 450 µl of 100% ethanol. The sample was mixed, allowed to precipitate overnight at -20°C and centrifuged for ten minutes at 10 000 g. The supernatant solution was removed, the pellet washed with 200 µl of 85% ethanol, dried and resuspended in 10 µl H<sub>2</sub>O. A 3 µl aliquot was

used in a reamplification PCR reaction in the presence of 1x PCR buffer, 20  $\mu$ M dNTP's, 0.2  $\mu$ M arbitrary primer and 0.2  $\mu$ M oligonucleotide HT<sub>11</sub>C and 2 units AmpliTaq polymerase using the same cycling parameters as the PCR reaction above. The resulting product was then used as probe in

5 a Northern hybridization assay to confirm regulation and cloned into a bacterial plasmid for sequence analysis.

*Fragment cloning*

Band 6a was cloned using the TA cloning kit (Invitrogen, Carlsbad CA). After lysis, colonies were screened by PCR for the correct insert size. Colonies were lysed in 20 mM Tris-HCl, pH 8, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 0.5% Tween 20 and 100  $\mu$ g/ml Proteinase K by incubating for 30 min at 56°C then 10 min at 99°C to inactivate the Proteinase K. Of this reaction 2 $\mu$ l was used in a PCR reaction of 20 mM Tris-HCl, pH 8, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 75  $\mu$ M dNTPs, 375 nM M-13 forward and reverse primers and 2.5 U of Taq polymerase (Gibco BRL, Gaithersburg MD). The reactions were cycled as follows: 95°C for 30 sec; 64°C for 30 sec; 72°C for 45 sec for 30 cycles. One clone, designated 6a.2, was chosen for sequencing.

*Fragment sequencing*

20 Clone 6a.2 was sequenced according to the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase using the recommended protocol from Applied Biosystems (Foster City CA). Spin columns (AGTC) were employed to remove

unincorporated dye-labeled nucleotides after cycle sequencing. Automated DNA sequencing grade 4.75% polyacrylamide gels were run for all the DNA sequencing samples using ABI 373 DNA sequencers. Sequencing data was edited using Sequence Navigator and assembled using DNASTar

5 (DNASTar, Madison WI). The sequence was trimmed of RADE primers and used in a BLAST search using Millennium Software (Boston MA). A nucleotide homology search of clone 6a.2 sequence revealed a 98% identity with human MT-II-. Over the coding sequence, however, there were no amino acid mismatches (Figure 3)

10 Confirmation of regulation using Northern blots and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR):

To confirm the results obtained with the PCR amplification of cellular mRNAs, Northern blots and qRT-PCR were used to assess the effect of 17- $\beta$  estradiol or other compounds on message levels. Unless

15 otherwise noted, all experiments use cells transiently overexpressing hER $\beta$ L in response to adenovirus infection as described above, and were treated with compound for 24 hours. In some cases cells transiently overexpressing ER- $\alpha$  were used for comparison. Methods for adenovirus-mediated overexpression of ER- $\alpha$  are identical to those described for ER- $\beta$ .

20 Northern blots

Poly A + RNA was isolated from total RNA using Oligotex mRNA Isolation kit (Qiagen, Hilden Germany) according to manufacturers instructions. Six micrograms of mRNA or 10  $\mu$ gs of total RNA were

separated on a 1.5% agarose, 0.22 M formaldehyde, 10 mM HEPES, 1 mM EDTA gel. RNA was transferred to Hybond-N (Amersham Pharmacia, Piscataway NJ) by capillary action in 20X SSC (Gibco BRL, Gaithersburg MD ) overnight. After transfer, the membrane was UV-cross-linked and 5 dried at 80°C for 10 min. Northern Blots were pre-hybridized in Rapid Hyb solution (Amersham Pharmacia, Piscataway NJ) for 30 minutes.

The insert from clone 6a.2 was isolated from the plasmid using PCR as described above for colony screen. The PCR product was purified from an agarose gel using Wizard Preps (Promega, Madison WI). Re-10 amplified RADE fragments for band 6a or fragment from clone 6a.2 were random primer labeled using Redi-prime kit (Amersham Pharmacia, Piscataway NJ) according to manufacturers instructions. Unincorporated nucleotides were removed using a Nap-5 column (Amersham Pharmacia, Piscataway NJ) and incorporation of [<sup>32</sup>P]-dCTP measured by liquid 15 scintillation counting.

The probes were denatured at 100°C for 10 min and 1.5 x 10<sup>6</sup> CPM/ml of Rapid-Hyb hybridization solution was added to the membrane. The blots were hybridized at 65°C for 5 hours and were washed as follows: Once in 2X SSC, 0.1% SDS at 65° C for 15 min; twice in 0.2X 20 SSC, 0.1% SDS at 65°C for 15-30 min. Blots were exposed to film and to a PhosphorImager screen (Molecular Dynamics, Sunnyvale CA). After probing with 6a RADE fragment or 6a.2 cloned fragment, blots were probed with a cDNA homologous to GAPDH as above. Hybridization

signal from 6a.2 fragment was normalized to that of GAPDH on a PhosphorImager (Molecular Dynamics, Sunnyvale CA) to determine fold induction.

*QRT-PCR*

5 A MT-II fragment identical to clone 6a.2 except that it contained a 63 bp deletion was subcloned into pcDNA.3 (Invitrogen, Carlsbad CA). RNA was transcribed using the T7 Promoter Large Scale Transcription Kit (Novagen, Madison WI). After phenol-chloroform extraction and ethanol precipitation the synthesized RNA was quantified and analyzed using UV  
10 spectrophotometry and gel electrophoresis.

Reverse transcription reactions were performed on 200 ng and 300 ng of DNased Saos-2 total RNA plus a known amount of MT-II standard RNA in a 20  $\mu$ l reaction. The reaction contained 1x PCR Buffer (Gibco BRL, Gaithersburg MD), 3.75 mM MgCl<sub>2</sub>, 1.25  $\mu$ M MT-II-specific reverse  
15 primer (5'-GGAATATAAGCAAACGGTCAGGGTC-3'), 0.5 mM dNTPs, 1 mM DTT, 20 units of RNasin (Promega) and 200 units of Superscript II Reverse Transcriptase (Gibco BRL, Gaithersburg MD). Reactions were incubated at 42°C for 15 minutes followed by 5 minutes at 99°C and 5 minutes on ice prior to amplification

20 PCR was initiated by adding 80 $\mu$ l of master mix containing MT-II specific forward primer (5'-GGCTCCTGCAAATGCAAAGAG-3') directly to the 20 $\mu$ l reverse transcriptase reaction. Final concentration of reagents in the 100 $\mu$ l PCR reaction was as follows: 0.25  $\mu$ M each MT-II -specific

primer, 1x PCR Buffer (Gibco BRL, Gaithersburg MD), 0.1 mM dNTPs, 1.5 mM MgCl<sub>2</sub> and 0.5 units of Taq DNA Polymerase (Gibco BRL, Gaithersburg MD). Two step PCR was carried out in a PE 9600 (Perkin-Elmer, Norwalk CT) for 40 cycles as follows: 95°C for 30 sec, 64°C for 5 min. Samples were incubated at 64°C for 10 min after amplification.

PCR products were separated and analyzed on a reverse-phase ion-pair high performance liquid chromatography DNASep column (Sarasep, San Jose CA). The elution system was a gradient of acetonitrile in 0.1M triethylammonium acetate (Fluka, Ronkonkoma NY) at a flow rate of 0.7 ml/min. The acetonitrile gradient increased from 14.6% to 16.6% over 5 minutes. The amount of product from the standard and the native RNA was determined by UV absorbance detection at 254 nm and signal was analyzed by an on-line integrator. From the chromatograms, the ratio of the area under each peak was used to determine the ratio of the amount 10 of input MT-II standard RNA to the amount of native MT-II message in the 15 Saos-2 RNA.

In LNCaPLN3 cells, the magnitude of MT-II induction by 17-β estradiol is approximately 6 fold (Figure 4). In Saos-2 cells, 10 nM 17-β estradiol upregulates MT-II mRNA as much as 14 fold (Figure 4). This 20 upregulation in Saos-2 cells has been repeated in at least 20 experiments and the range of induction is 3.5-14 fold. The EC<sub>50</sub> of this regulation is 4.6 ± 2.7 nM as assessed by qRT-PCR (Figure 5). If Saos-2 cells are treated with 10 nM 17-β-estradiol and RNA prepared at different times

post treatment, the first discernible increase in MT-II message occurs after 8 hours and the response peaks by 24 hours (Figure 6).

Since the samples prepared for differential display overexpressed hER $\beta$ L, , MT-II regulation was assessed in native Saos-2 cells and those 5 overexpressing ER $\alpha$ . As illustrated in Figure 7 , endogenous levels of ER- $\beta$  or overexpressed levels of ER- $\alpha$  were ineffective at mediating 17 $\beta$ -estradiol's regulation of MT-II.

Other compounds were tested for their ability to upregulate MT-II in Saos-2 cells. Ten nanomolar diethylstilbestrol (Sigma, St. Louis MO) 10 and 0.1  $\mu$ M genistein (RBI, Natick MA) both increased MT-II mRNA. One micromolar of the antiestrogen, ICI-182780 (Zeneca, Wilmington DE), completely blocked induction by 17 $\beta$ -estradiol and genistein but had no effect when given alone (Figure 8). Co-treatment with 1 $\mu$ M of the antiprogestrone/antiglucocorticoid, RU486 (Ligand Pharmaceuticals, La 15 Jolla CA), did not block regulation by 17 $\beta$ -estradiol (data not shown).

**EXAMPLE 4: TREATMENT OF SAOS-2 CELLS WITH CYCLOHEXIMIDE**

To determine if the increase in MT-II mRNA requires new protein synthesis, cycloheximide was used after hER $\beta$ L overexpression and 20 before treatment with 17- $\beta$  estradiol to arrest translation.

Verification of cycloheximide's effect on protein synthesis

Cells were plated and infected with hER $\beta$ L virus as described above, then pre-treated with 10  $\mu$ g/mL of cycloheximide or vehicle for one

hour at 37°C. After this preincubation, 10 µg/mL of cycloheximide, 10 nM 17-β estradiol and 50 µCi/ml <sup>35</sup>S-Methionine (NEN, Boston MA) in methionine-deficient media was added and incubation continued at 37°C for 8 or 24 hours. Cells were washed 3x in cold PBS and then scraped \ 5 off plates in 500 µl of PBS. Cells were pelleted and resuspended in 200 µl of RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, and 50 mM Tris, pH 8.0). Methionine incorporation was measured by TCA precipitation. Five and ten microliters of each sample were spotted on individual Whatman filters. Filters were boiled in 10% trichloroacetic acid 10 for 10 minutes, then washed 3 times for 10 minutes in deionized water, 3 times for 10 minutes in 95% ethanol and once for 10 minutes in acetone. The dried filters were placed in scintillation fluid and counted for 1 minute.

Treatment of cells with 17-β estradiol

15 Cells were plated and infected with hER $\beta$ L virus as described above. Cells were pretreated with 10 µg/mL of cycloheximide or vehicle for one hour at 37°C. After this preincubation, 10 nM 17-β estradiol or vehicle was added and incubation continued at 37°C for 8 or 24 hours. RNA was isolated from the cells as described above and gene expression 20 evaluated by Northern blot analysis or qRT-PCR as described in Example 3.

Verification of functional ER-β protein after cycloheximide treatment

Duplicate cultures were prepared and treated as described in the previous section. After the 8 hour incubation, cells were washed four times with DMEM to remove 17- $\beta$  estradiol. Cycloheximide (10  $\mu$ g/mL) was added to all samples as was 1 nM [ $^3$ H]-estradiol. Some cells were 5 co-treated with 0.3  $\mu$ M diethylstilbestrol to estimate nonspecific binding.

After incubation for 2.5 hr at 37°C, cells were washed with DMEM and lysed with 0.1% sodium dodecyl sulfate. DPM were measured by liquid scintillation counting.

The MT promoter contains glucocorticoid and metal response 10 elements, but EREs have not been described. It is possible the effect of 17- $\beta$  estradiol on MT-II mRNA expression is indirect. After overexpression of ER- $\beta$ , Saos-2 cells were treated with cycloheximide to severely limit new protein synthesis (Figure 9A). Although comparable amounts of receptor protein were expressed with and without 15 cycloheximide treatment as measured by a whole cell binding assay (Figure 9B), MT-II induction did not occur (Figure 9C,D).

**EXAMPLE 5 : TREATMENT OF RATS WITH 17- $\beta$  ESTRADIOL**

Because 17- $\beta$  estradiol upregulates MT-II in LNCaPLN3 cells, a 20 prostate cancer cell line, we looked for a similar response in the rat prostate.

All animals were treated according to institutional guidelines using approved protocols. Adult (15-19 week, ~375g) castrated Sprague-

Dawley rats were purchased from Taconic Farms (Germantown NY). Ten days after castration, rats were injected with vehicle, 16 µg 17- $\beta$  estradiol, 16 µg diethylstilbestrol (Sigma, St. Louis MO), or 16 µg 17- $\beta$  estradiol plus 1.6 mg raloxifene (synthesized in-house) subcutaneously 5 once per day for three days. Approximately 24 hr after the last dose, rats were euthanized by CO<sub>2</sub> asphyxiation and the prostate excised. Total RNA was prepared and analyzed for MT-II mRNA as described above. In another experiment, rats were dosed for 1, 2 or 3 days with 16 µg 17- $\beta$  estradiol before prostate tissue was collected.

10           Metallothionein-II mRNA increased five-fold after 17- $\beta$  estradiol treatment. A similar response occurred when rats were dosed with diethylstilbestrol, but this upregulation was blocked by coadministration with a 100 fold excess of raloxifene hydrochloride, an estrogen antagonist (Figure 10). Although the initial experiments used tissue from 15 rats dosed for three days, the upregulation of MT-II in the rat prostate is first seen after two days of dosing with 17- $\beta$  estradiol (Figure 11).

**EXAMPLE 6 : ERE-LUCIFERASE REPORTER ASSAY USING MCF-7 CELLS**

Because MT-II regulation in Saos-2 cells is mediated by ER- $\beta$  and not ER- $\alpha$ , another assay was needed to compare selectivity of compounds 20 for these two receptors in cell based assays. MCF-7 is an estrogen-responsive breast cancer cell line and, in our hands, expresses only ER- $\alpha$  as measured by RT-PCR (data not shown). When MCF-7 cells are

transiently infected with an ERE-reporter gene construct and treated with 17- $\beta$  estradiol, reporter gene activity can be measured.

MCF-7 HTB 22, ATCC , Manassas VA) cells are passaged twice a week with growth medium [D-MEM/F-12 medium containing 10% (v/v)

5 heat-inactivated fetal bovine serum100 U/ml penicillin g, 100  $\mu$ g/ml streptomycin sulfate, 2 mM GlutaMAX-1 ]. The cells are maintained in vented flasks at 37°C inside a 5% CO<sub>2</sub>/95% humidified air incubator.

One day prior to treatment, the cells are plated with growth medium at 25,000/well into 96 well plates and incubated at 37°C overnight.

10 The cells are infected for 2 hr at 37°C with 50  $\mu$ l/well of a 1:10 dilution of adenovirus 5-ERE-TK-luciferase (Bodine et. al., 1997) in experimental medium [phenol red-free DMEM/F-12 medium containing 10% (v/v) heat-inactivated charcoal-stripped fetal bovine serum, 100 U/ml

penicillin g, 100  $\mu$ g/ml streptomycin sulfate, 2 mM GlutaMAX-1 , 1 mM 15 sodium pyruvate]. The wells are then washed once with 150  $\mu$ l of

experimental medium. Finally, the cells are treated for 24 hr at 37°C in replicates of 8 wells/treatment with 150  $\mu$ l/well of vehicle ( $\leq$  0.1% v/v DMSO) or compound that is diluted  $\geq$  1000-fold into experimental medium.

20 After treatment, the cells are lysed on a shaker for 15 min with 25  $\mu$ l/well of 1X cell culture lysis reagent (Promega, Madison WI). The cell lysates (20  $\mu$ l) are transferred to a 96 well luminometer plate, and

luciferase activity is measured in a MicroLumat LB 96 P luminometer (EG & G Berthold, Bad Wildbad Germany) using 100 µl/well of luciferase substrate (Promega, Madison WI). Prior to the injection of substrate, a 1 second background measurement is made for each well. Following the 5 injection of substrate, luciferase activity is measured for 10 seconds after a 1 second delay. After subtracting background subtracts the mean and standard deviation are calculated.

Compounds which induce MT-II in Saos-2 cells and do not stimulate reporter gene activity in MCF-7 cells are thus selective for ER-β.

10 In addition, results from these two assays can also be used to select a compound that is selective for ER-α as shown in Figure 12.

#### Discussion

Using differential display, it was unexpectedly discovered that ER-β increases MT-II mRNA in two cell lines treated with 17-β estradiol. To our knowledge, this is the first gene discovered to be regulated by this new form of the ER. This response has been extensively characterized in the human osteosarcoma cell line Saos-2. A variety of estrogens can upregulate MT-II, and this response is blocked by cotreatment with the 15 estrogen antagonist ICI-182780. The EC<sub>50</sub> for 17-β estradiol is approximately 5 nM and this response is mediated by ER-β acting through 20 as yet unknown proteins.

The action of ER- $\beta$  on MT-II is not a general phenomenon as a survey of over a dozen cell lines failed to reveal this induction in samples other than Saos-2 and LNCaPLN3 cells. However, the fact that MT-II is regulated by 17- $\beta$  estradiol in the rat prostate strengthens the possibility

5 that this is a physiologically relevant response and not a artifact of receptor overexpression in cancer cell lines. We are confident that this prostate response is mediated by the ER for two reasons: A nonsteroidal estrogen (diethylstilbestrol) upregulates MT-II and raloxifene, an estrogen agonist/antagonist, blocks 17- $\beta$  estradiol's action. However, at this time

10 it is not clear if this induction reflects activity of ER- $\beta$  and/or ER- $\alpha$ .

Although ER- $\beta$  is the predominant form of the ER in the rat prostate, ER- $\alpha$  is also present. Current studies are underway to define the receptor type responsible for this in vivo response.

Metallothioneins are low molecular weight, cysteine rich proteins

15 that bind metals such as cadmium, copper and zinc. Although the first metallothionein was discovered over forty years ago (Vallee, 1957), debate continues as to their function. Several proposals have been made and these include protection from metal toxicity, regulation of zinc and copper homeostasis and defense against oxidative stress. Regulation of

20 energy balance has also been implicated because, after reaching sexual maturity, MT (-I and -II) knockout mice become obese (Beattie et al, 1998).

Recently, studies have detailed how MT may act to regulate zinc homeostasis in cells. Using purified zinc-dependent enzymes such as *E. coli* alkaline phosphatase, bovine carboxypeptidase A and sheep sorbitol dehydrogenase two recent publications show how agents in the cellular milieu can facilitate exchange between zinc complexed with MT and (metal depleted) apoenzymes (Jacob et al, 1998; Jiang et al, 1998). Citrate and glutathione can influence the direction of zinc transfer and thus regulate enzyme activity depending on the redox state of the cell. Although not an enzyme, the ER also requires zinc for activity, and ER from MCF-7 cells can reversibly exchange zinc with purified MT in vitro (Cano-Gauci et al, 1996).

A myriad of agents can regulate MT levels, including glucocorticoids and metals such as cadmium (for review see Hamer, 1986). Estrogens are not a classical regulators of MT, but two intriguing papers appear in the literature. First, a two week treatment of female rats with 17- $\beta$  estradiol upregulated a copper binding protein in intestinal mucosa which reduced the amount of copper absorbed into the plasma. Since the molecular weight of this protein was about 10K, the authors suggest it is MT (Cohen et al, 1979). Recently, MT was identified in a subtractive hybridization screen of uterine mRNAs regulated after a single injection of 17- $\alpha$  ethinyl estradiol (Rivera-Gonzalez et al, 1998). Although the isoform is not identified, a MT transcript increased three fold between four and eight hours after 17- $\alpha$  ethinyl estradiol stimulation. In addition,

it is not clear which receptor type effects this regulation because ER- $\alpha$ , not ER- $\beta$ , is the most abundant ER in the uterus (Kuiper et al, 1996; Couse et al, 1997).

Since the function of MT is only beginning to be elucidated and is  
5 completely unknown for ER- $\beta$ , understanding the significance of their association is impossible at this time. However, even if the connection between these two proteins is unclear, the observation of regulation can still be exploited to help design an ER- $\beta$  or ER- $\alpha$  specific ligand. The fact that genistein upregulates MT-II- in Saos-2 cells as well or better than  
10 17 $\beta$ -estradiol indicates an ER- $\beta$ -selective compound can effectively direct transcription. In addition, several structurally diverse compounds which bind to ER- $\beta$  can also upregulate this message in Saos-2 cells. When data on a compound's ability to regulate MT-II in Saos-2 cells are coupled with information about how it regulates reporter gene activity in an MCF-7 cell,  
15 ER- $\beta$  and ER- $\alpha$  selectivity can be assessed. Thus, when used together, these two assays are tools to help design selective compounds for either ER type. Finally, if the regulation in the prostate can also be shown to be mediated by ER- $\beta$ , then the in vivo activity of compounds can be assessed, providing another valuable tool for drug discovery.

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What is claimed is:

1. A method for screening a test compound that binds to an ER in a receptor binding assay, wherein said method detects ER- $\beta$  polypeptide-mediated transcription, said method comprising the steps of:
  - (a) providing a cell which comprises at least one estrogen-regulated DNA sequence encoding MT-II and at least one DNA sequence encoding ER- $\beta$  polypeptide, wherein said receptor is transcriptionally active;
  - (b) contacting said cell with either said test compound which binds ER or a control; and
  - (c) detecting the expression of said MT-II, wherein enhanced expression of said MT-II relative to a control indicates that said test compound has estrogen agonist activity.
- 15 2. The method of claim 1, wherein the DNA sequence encoding ER- $\beta$  polypeptide is incorporated into an adenovirus.
3. The method of claim 2 wherein the adenovirus is a replication-defective Ad5 virus.
- 20 4. The method of claim 1, wherein said cells endogenously express ER- $\beta$  mRNA at a higher rate than ER- $\alpha$  mRNA.

5. The method of claim 4, wherein said cells are transformed with a recombinant DNA plasmid comprising a polynucleotide encoding a human ER- $\beta$  operably linked to a suitable promoter, wherein said transformed cells express human ER- $\beta$  at higher levels than the cells which have not 5 been transformed.

6. The method of claim 1 wherein said cells are Saos-2 or LNCaPLN3 cells.

10 7. The method of claim 1, wherein said cells do not have a functional ER- $\alpha$ .

8. A method for screening a test compound that binds to an ER in a receptor binding assay, wherein said method detects inhibition of ER- $\beta$  15 polypeptide-mediated transcription, said method comprising the steps of:  
(a) providing a cell which comprises at least one estrogen DNA sequence encoding MT-II and at least one DNA sequence encoding ER- $\beta$  polypeptide, wherein said receptor is transcriptionally active;  
(b) contacting said cell with one or more estrogens in the 20 presence of the test compound known to bind ER; and  
(c) detecting the expression of said MT-II, wherein decreased expression of said MT-II relative to the addition of one or more estrogens alone indicates that said test compound has estrogen antagonist activity.

9. The method of claim 8, wherein the DNA encoding ER- $\beta$  polypeptide is incorporated into an adenovirus.

5 10. The method of claim 9 wherein the adenovirus is a replication-defective Ad5 virus.

11. The method of claim 8, wherein said cells endogenously express ER- $\beta$  mRNA at a higher rate than ER- $\alpha$  mRNA.

10

12. The method of claim 11, wherein said cells are transformed with a recombinant DNA plasmid comprising a polynucleotide encoding a human ER- $\beta$  operably linked to a suitable promoter, wherein said transformed cells express human ER- $\beta$  at higher levels than the cells which have not  
15 been transformed.

13. The method of claim 12 wherein said cells are Saos-2 or LNCaPLN3 cells.

20 14. The method of claim 13, wherein said cells do not have a functional ER- $\alpha$ .

15. A method of screening test compounds to identify drug candidates which mimic estrogen's effect on ER- $\beta$ - or ER- $\alpha$ -mediated transcription, said method comprising the steps of:

(a). contacting said test compound with a plurality of:

5 (i) first cells comprising at least one endogenous DNA sequence encoding MT-II and at least one DNA sequence encoding a ER- $\beta$  polypeptide, wherein said receptor is transcriptionally active and

(ii) second cells comprising an ERE reporter gene construct, wherein said cells express ER- $\alpha$  polypeptide;

10 (b) identifying compounds which increase expression of MT-II in said first cells relative to control but have minimal effect on expression of said reporter gene in said second cells, wherein said compounds are considered ER- $\beta$  selective; or

15 (c) identifying compounds which increase expression of the reporter gene in said second cells relative to control but have minimal effect on expression of MT-II in said first cells, wherein said compounds are considered ER- $\alpha$  selective.

20 16. The method of claim 15, wherein the DNA encoding ER- $\beta$  polypeptide of said first cells is incorporated into an adenovirus.

17. The method of claim 16 wherein the adenovirus is a replication-defective Ad5 virus.

18. The method of claim 15, wherein said first cells endogenously  
5 express ER- $\beta$  mRNA at a higher rate than ER- $\alpha$  mRNA.

19. The method of claim 15, wherein said first cells are transformed  
with a recombinant DNA plasmid comprising a polynucleotide encoding a  
human ER- $\beta$  operably linked to a suitable promoter, wherein said  
10 transformed first cells express human ER- $\beta$  at higher levels than said  
second cells.

20. The method of claim 15 wherein said first cells are Saos-2 or  
LNCaPLN3 cells.

15

21. The method of claim 15, wherein said first cells do not have a  
functional ER- $\alpha$  and said second cells do not have a functional ER- $\beta$ .

22. The method of claim 15, wherein said second cells were  
20 transformed with a DNA polynucleotide comprising an ERE operably  
linked to a reporter gene.

23. The method of claim 22, wherein said reporter gene is a luciferase gene.

24. A method of screening test compounds to identify drug candidates which inhibit estrogen's effect on ER- $\beta$ - or ER- $\alpha$ -mediated transcription, said method comprising the steps of:

- contacting said test compound in the presence and absence of one or more estrogens with a plurality of:
  - first cells comprising at least one endogenous DNA sequence encoding a metallothionein (MT-II) gene and at least one DNA sequence encoding a ER- $\beta$  polypeptide, wherein said receptor is transcriptionally active; and
  - second cells comprising an ERE-reporter gene construct, wherein said cells express ER- $\alpha$  polypeptide; and
- identifying compounds which decrease expression of MT-II in said first cells relative to treatment with one or more estrogens but have minimal effect on expression of said reporter gene in said second cells, wherein said compounds are considered ER- $\beta$  selective; or
- identifying compounds which decrease expression of the reporter gene in said second cells relative to treatment with one or more estrogens but have minimal effect on expression of MT-II in

said first cells, wherein said compounds are considered ER- $\alpha$  selective.

25. The method of claim 24, wherein the DNA encoding ER- $\beta$  5 polypeptide of said first cells is incorporated into an adenovirus.

26. The method of claim 25 wherein the adenovirus is a replication-defective Ad5 virus.

10 27. The method of claim 24, wherein said first cells endogenously express ER- $\beta$  mRNA at a higher rate than ER- $\alpha$  mRNA.

28. The method of claim 24, wherein said first cells are transformed with a recombinant DNA plasmid comprising a polynucleotide encoding a 15 human ER- $\beta$  operably linked to a suitable promoter, wherein said transformed first cells express human ER- $\beta$  at higher levels than said second cells.

29. The method of claim 24 wherein said first cells are Saos-2 or 20 LNCaPLN3 cells.

30. The method of claim 24, wherein said first cells do not have a functional ER- $\alpha$  and said second cells do not have a functional ER- $\beta$ .

31. The method of claim 24, wherein said second cells were transformed with a DNA polynucleotide comprising an ERE operably linked to a reporter gene.

5

32. The method of claim 31, wherein said reporter gene is a luciferase gene.

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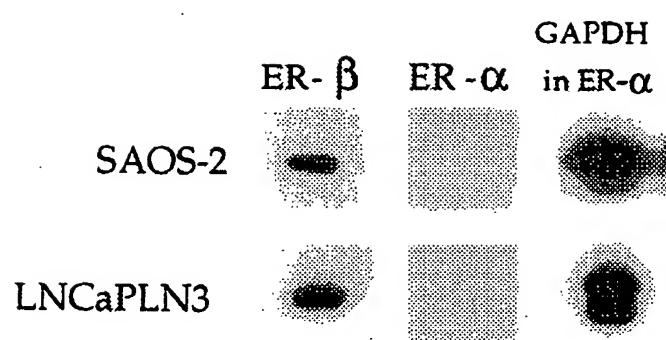


FIG.1

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Saos-2 LNCaPLN3

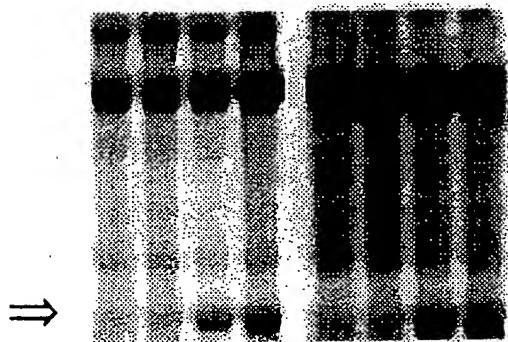


FIG.2

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Clone 6a:	1	ATCCCCAACTGCTTCTGGCGCCGGTGAECTCCTGCACCTGGCCGGCTCCTGCAAATGCA	60
MT-II	5	ACCCCCAACTGCTGTGGCGCCGGTGAECTCCTGCACCTGGCCGGCTCCTGCAAATGCA	64

Clone 6a:	61	AAGAGTGCAAATGCACCTCTGCAAGAAAAGCTGCTGCTGCCTGTGGCTGTG	120
MT-II	65	AAGAGTGCAAATGCACCTCTGCAAGAAAAGCTGCTGCTGCCTGTGGCTGTG	124

Clone 6a:121	CCAAGTTGCCAGGGCTGCATCTGCAAAGGGGGTGGACAAGTGCAGCTGCTGGCCT	180	
MT-II:	125	CCAAGTTGCCAGGGCTGCATCTGCAAAGGGGGTGGACAAGTGCAGCTGCTGGCCT	184

Clone 6a:181	GATGCTGGACAGCCCC	196	
MT-II	185	GATGCTGGACAGCCCC	200

**FIG. 3A**

MDPNCSAACGDSCTCAGSCKCKECKTSCKKSCCCPVGCAKCAQGCICKGASDKCSCCA\*

**FIG. 3B**

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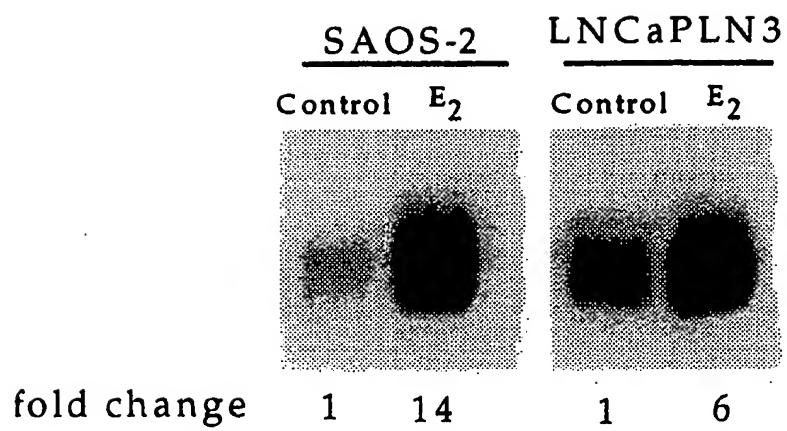


FIG.4

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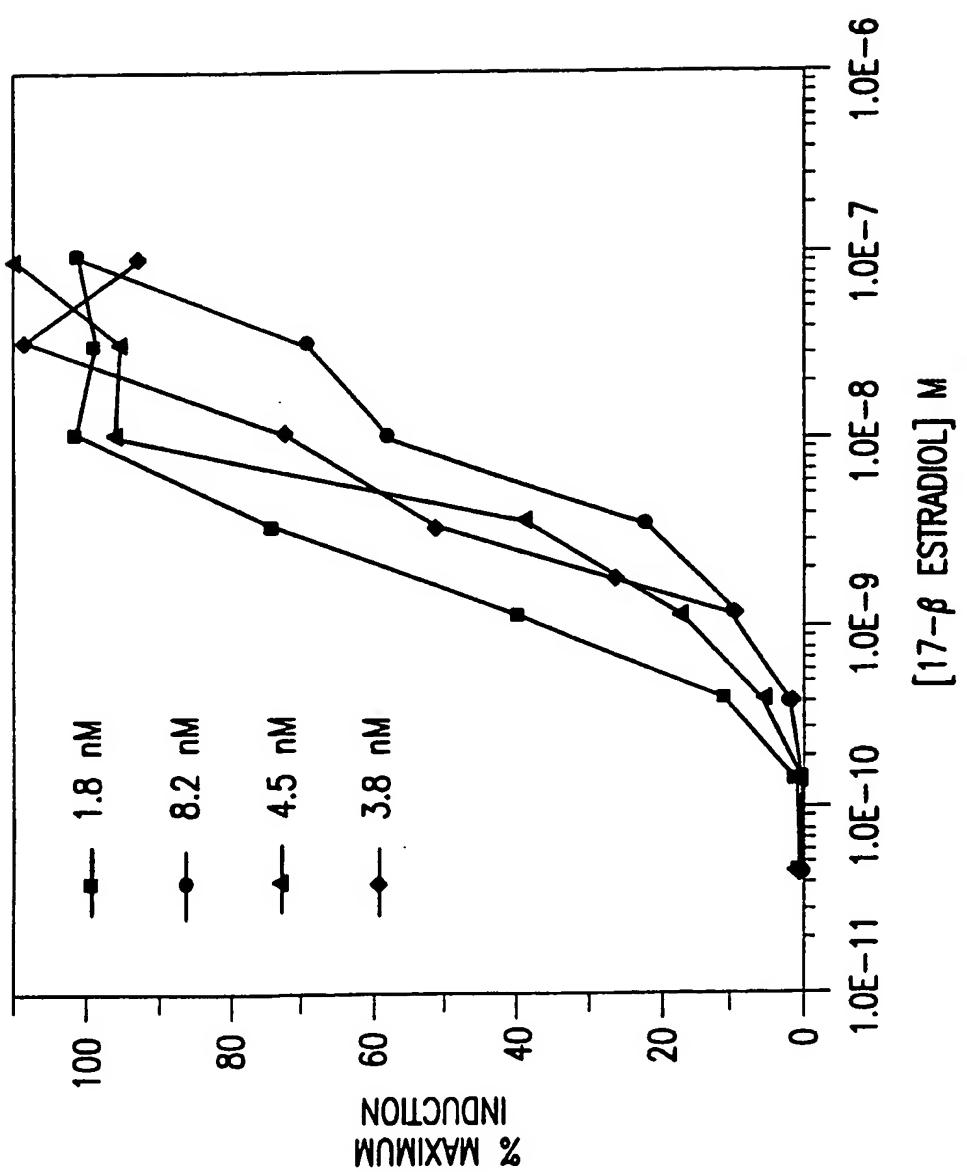


FIG. 5

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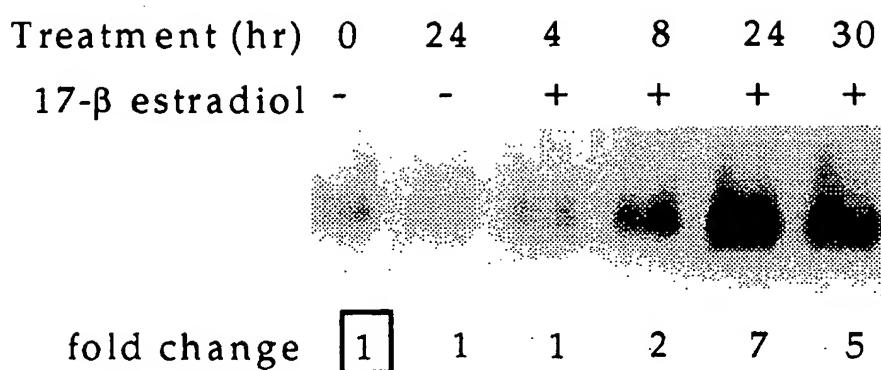


FIG.6

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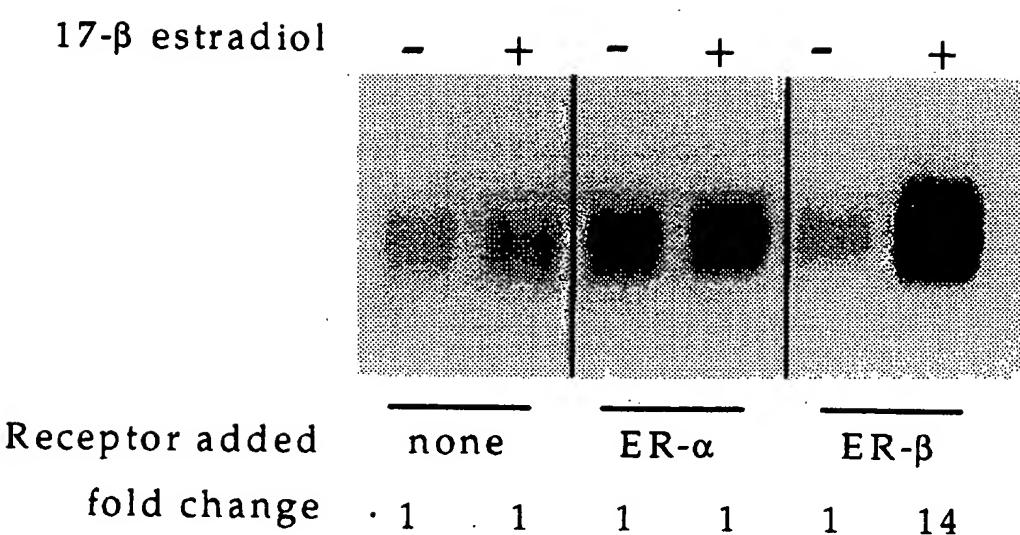


FIG. 7

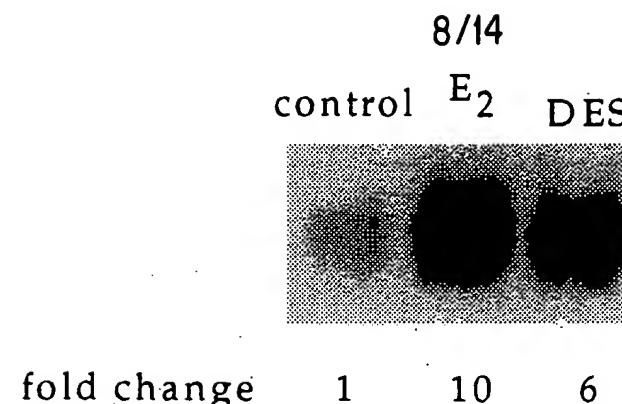


FIG.8A

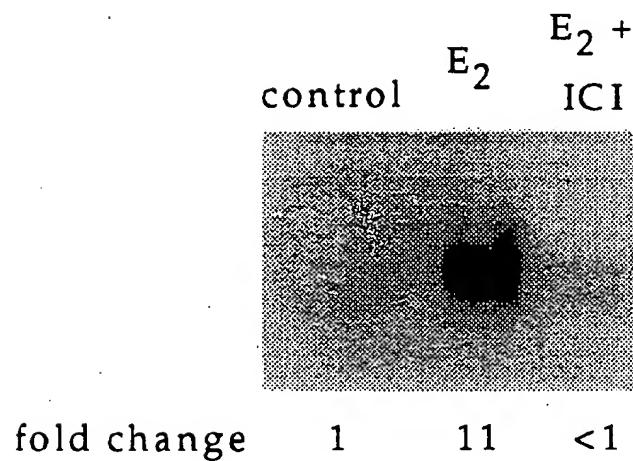


FIG.8B

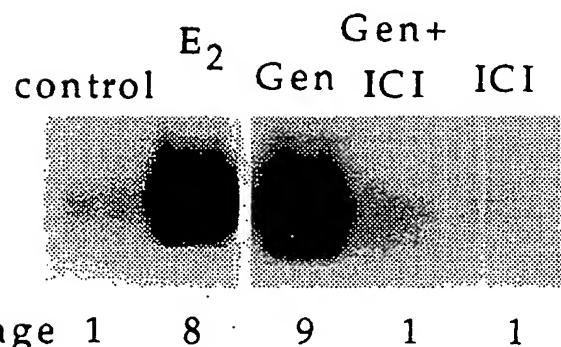


FIG.8C

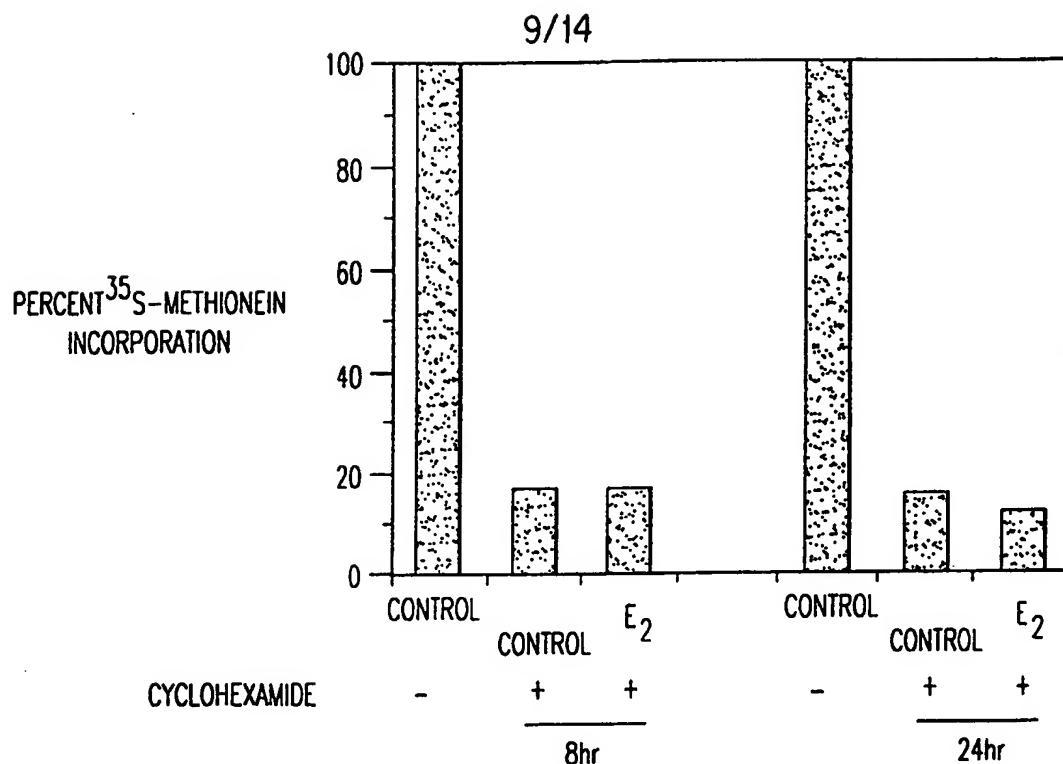


FIG.9A

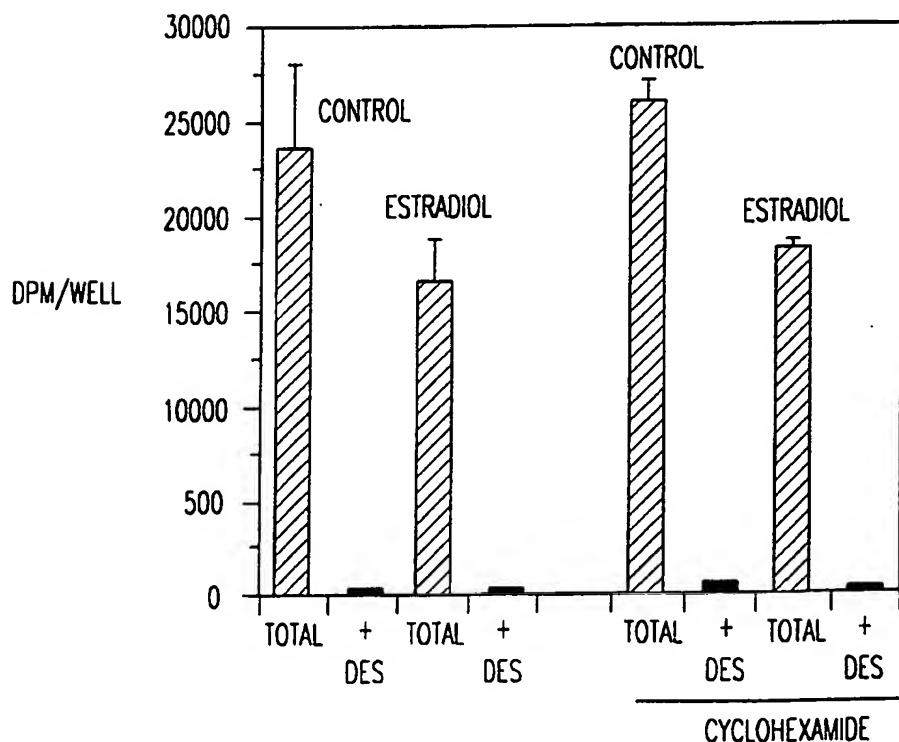


FIG.9B

SUBSTITUTE SHEET (RULE 26)

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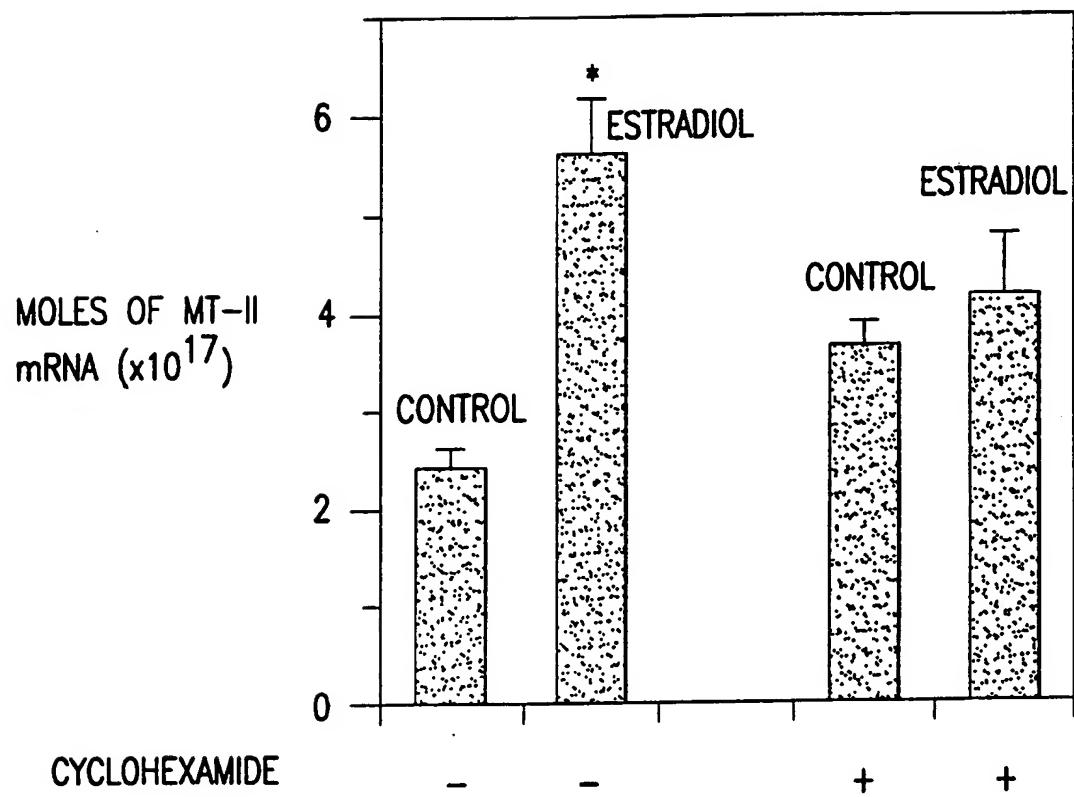


FIG.9C

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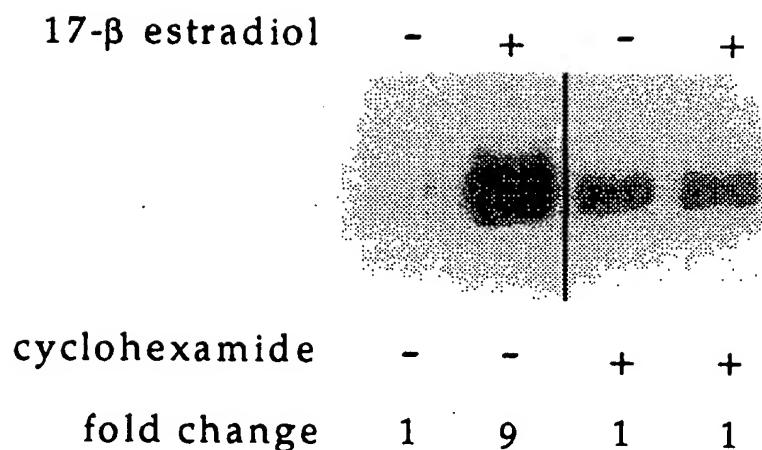


FIG.9D

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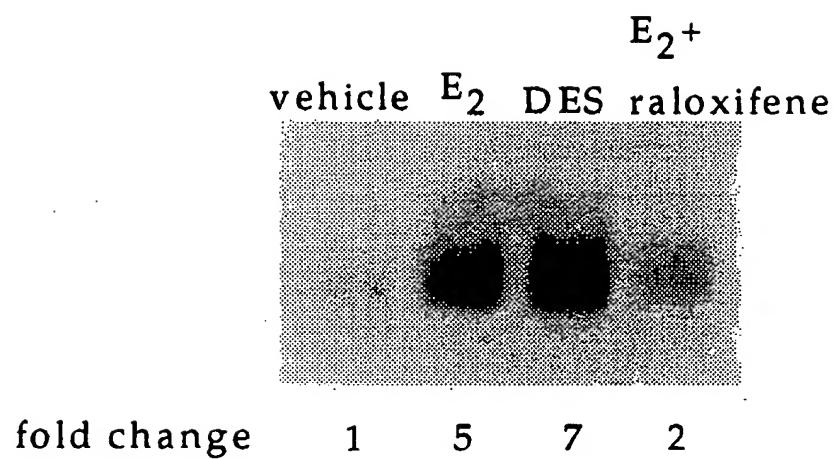


FIG. 10

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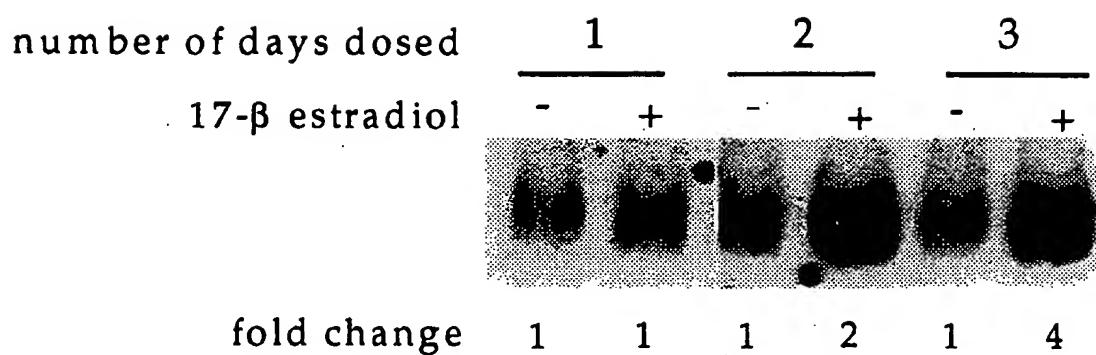


FIG. 11

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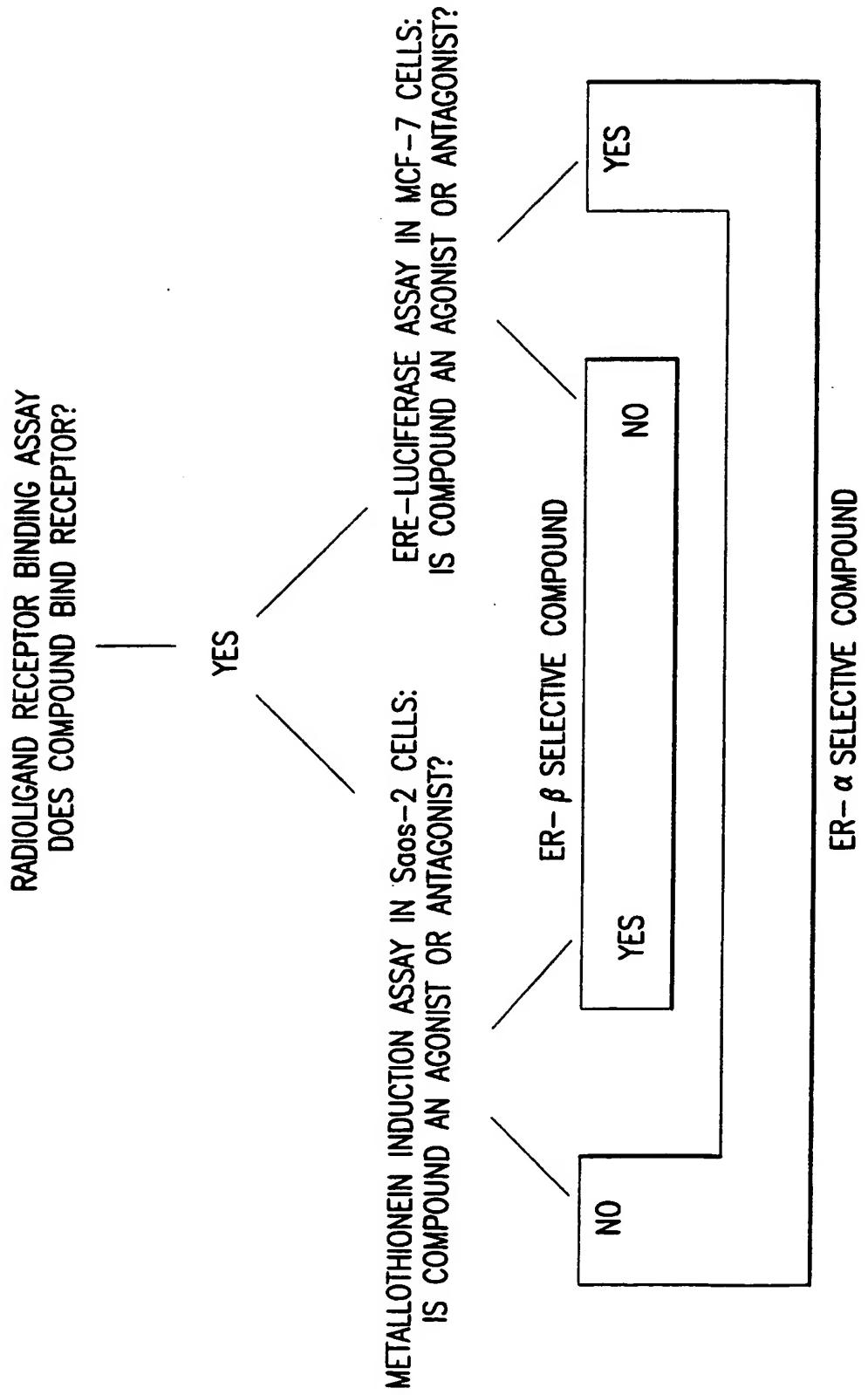


FIG. 12

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/29856

**A. CLASSIFICATION OF SUBJECT MATTER**  
**IPC 7 C12Q1/68 G01N33/50**

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

**IPC 7 C12Q G01N**

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>COLDHAM N.G. ET AL.: "Evaluation of a recombinant yeast cell estrogen screening assay"  <b>ENVIRON HEALTH PERSPECTIVES</b>,  vol. 105, no. 7, - July 1997 (1997-07)  pages 734-742, XP000911153  the whole document</p> <p>—</p>	
A	<p>MCDONNELL D P ET AL: "NUCLEAR HORMONE RECEPTORS AS TARGETS FOR NEW DRUG DISCOVERY"  BIO/TECHNOLOGY, US, NATURE PUBLISHING CO.  NEW YORK,  vol. 11, 1 November 1993 (1993-11-01),  pages 1256-1261, XP002018219  ISSN: 0733-222X  the whole document</p> <p>—</p> <p>—/—</p>	

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the International filing date but later than the priority date claimed

"T" later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the International search

Date of mailing of the International search report

26 May 2000

07/06/2000

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Müller, F

## INTERNATIONAL SEARCH REPORT

Int'l Application No

PCT/US 99/29856

## C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 88 03168 A (SALK INST FOR BIOLOGICAL STUDI) 5 May 1988 (1988-05-05) see whole doc. esp. claims 28 ff. _____	
P, X	WO 99 11760 A (UNIV CALIFORNIA) 11 March 1999 (1999-03-11) the whole document _____	1-32

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/29856

Patent document cited in search report	Publication date		Patent family member(s)	Publication date
WO 8803168	A	05-05-1988	US 5071773 A AT 182173 T AU 616389 B AU 8238887 A DE 3752284 D DE 3752284 T EP 0287653 A EP 0733705 A JP 10004987 A JP 10108684 A JP 11137272 A JP 1500964 T US 5597705 A US 5534418 A US 5606021 A US 5312732 A US 5298429 A	10-12-1991 15-07-1999 31-10-1991 25-05-1988 19-08-1999 25-11-1999 26-10-1988 25-09-1996 13-01-1998 28-04-1998 25-05-1999 06-04-1989 28-01-1997 09-07-1996 25-02-1997 17-05-1994 29-03-1994
WO 9911760	A	11-03-1999	AU 8924398 A	22-03-1999